Biocompatibility of degradable biomaterials

Lam, King Hong

Chapter 5

MORPHOLOGY AND WETTABILITY ARE IMPORTANT FACTORS DETERMINING THE INFLAMMATORY RESPONSE AGAINST POLY(L-LACTIC ACID): A SEMI-QUANTITATIVE STUDY USING MONOCLONAL ANTIBODIES

K.H. Lam [1], J.M. Schakenraad [2], H. Groen [1], H. Esselbrugge [3], P.J. Dijkstra [3], J. Feijen [3] and P. Nieuwenhuis [1]

1) Department of Histology and Cell Biology, Section Biomaterials, Medical Faculty, University of Groningen, The Netherlands
2) Biomedical Technology Centre, Medical Faculty, University of Groningen, The Netherlands
3) Department of Chemical Technology, University of Twente, The Netherlands

Summary

In this study, the influence of morphology and wettability of both degradable and non- degradable polymer films on the inflammatory response was investigated. The cells involved in the inflammatory response were characterized with monoclonal antibodies (mAbs).

Films with a different morphology (non-porous, porous and "combi"(porous with a non porous layer on one side) were cast from poly(L-lactic acid). As controls, non-degradable polymer films (non-porous) polytetrafluoroethylene (PTFE) and (porous) expanded PTFE (ePTFE) were used. Strips of films of 15 x 2 mm and thicknesses ranging from 33 μm (non-porous PLLA film) to 1 mm (PTFE film) were subcutaneously implanted in female $(AOxBN)F_1$ rats. Prior to implantation, the contact angles were measured using H_2O (PLLA: 72°, PTFE: 101°) and α-bromonaphthalene (PLLA: 23°, PTFE: 66°) as wetting agents. The results indicate that PLLA is more wettable than PTFE.

An *in vitro* direct contact cytotoxicity test showed no leakage of large quantities of toxic products.

Histological examination, characterization and quantification of the cells involved in the inflammatory response were performed after 1, 3, 7, 14, 40, 90 and 180 days, using both conventional light microscopy and monoclonal antibodies against: all leucocytes (OX 1), neutrophilic granulocytes (HIS 48), T lymphocytes (OX 19), vast majority of B lymphocytes (HIS 40), vast majority of macrophages (ED 1), subsets of mature/resident macrophages (ED 2), subsets of macrophages (ED 3), natural killer (NK) cells/large granular lymphocytes

Submitted for publication to the Journal of Biomedical Materials Research

(LGL)(α-Asialo GM_1) and cells expressing MHC class II antigen (activated fibroblasts, dendritic cells) (HIS 19).

The results indicate that up till day 40 there was no large difference in the intensity of the inflammatory response against PLLA or PTFE (porous as well as non-porous). The inflammatory response observed can largely be considered as part of the wound healing reaction. Major differences in the tissue reaction became apparent from day 40 on.

From day 40 on, the PLLA films provoke a more intense inflammatory response as compared to the PTFE films. Using mAbs, it is shown that, generally, a higher number of leucocytes, T-lymphocytes, macrophages and activated fibroblasts surrounding the PLLA implant. There was hardly any B-lymphocyte activity against the PLLA and PTFE film from day 14 on, indicating that none of the implanted films induced the production of antibodies against them.

The porous side of the "combi" PLLA film provoked a more intense inflammatory response than the non-porous side. In general, the type and number of cells surrounding the PTFE and ePTFE films are the same as those localized in the subcutaneous tissue under the scar of the SHAM operation. Hardly any ingrowth of tissue was observed in the ePTFE films. In contrast, the inflammatory response against porous PLLA was mainly localized in the pores.

Using mAbs, different populations of macrophages involved in the inflammatory response surrounding the implant can be distinguished. The exact role of these subpopulations remains unclear.

In summary, it can be concluded that biodegradable PLLA films provoke a more intense inflammatory response than non degradable PTFE films due to their degradability and higher wettability. Porous PLLA provokes a more intense inflammatory response than non-porous PLLA. However, it is also demonstrated that differences in porosity only lead to differences in the inflammatory response, when wettability of a biomaterial is high enough.

5.1 Introduction

Increasing amounts of polymers are used as biomaterials in medical and dental materials and devices (1). Upon implantation of most polymers, local (1,2) and systemic (3) effects can be observed. The local tissue reaction or inflammatory response is a reaction of vascularized tissue to an injury. This reaction consists of a series of events occurring in a standard sequence. The inflammatory response aims at eliminating the cause of an injury, minimizing the damage and also trigger mechanisms for repairing the tissue damaged by injury. Variation of this standard pattern depends on the nature, the intensity and the localization of the injury, which, in the event of implantation of polymer as part of a biomaterial, is the result of the surgical procedure. However, in addition, implanted polymers themselves provoke an inflammatory response (2). Many investigators agree that for clinical application, this inflammatory response, caused by the implanted polymer, should be kept to a minimum. However, the intensity of an inflammatory response still acceptable for considering a polymer biocompatible remains a point of discussion and investigation. The quantification of this response and research on the factors determining the inflammatory response can lead to new strategies for minimizing the inflammatory response against implanted polymers.

One of the factors determining the inflammatory response is the **morphology** of the biomaterial. It is indicated in various studies that the morphology of the biomaterial is a factor influencing the inflammatory response (4,5). At the macroscopical level, Matlaga et al. demonstrated the role of shape (6). The intensity of the inflammatory response correlated positively with the number of edges of the implanted materials. At the microscopical level,

there is some indirect indication that the inflammatory response against implanted porous polymers or biomaterials appears to be more pronounced when compared to non-porous materials (7).

Wettability may influence the tissue reaction to the polymer, because there is a range in wettability which is optimal for cell adhesion, growth and spreading. Cells attach and proliferate less well on polymers having a wettability which is too low (8) or too high (9).

Another factor is **degradability**. Degrading polymers provoke a more intense inflammatory response compared to non-degrading polymers. A possible cause for this observation may be the release of monomers, oligomers and/or fragments upon degradation (10,11). However, at earlier stages of the degradation process, changes in the morphology of a polymer (film) may occur, which in turn may alter the inflammatory response.

Degradable and non-degradable polymer films, having a different morphology (porosity and wettability, were implanted subcutaneously in the back of the rat and the tissue reaction against them was evaluated. As biodegradable polymer, poly(L-lactic acid)(PLLA) was chosen. PLLA is a polymer with many (potential) uses (11,12,13). As non-degradable polymer, substantially differing in wettability, non-porous and porous polytetrafluoroethylene (PTFE and ePTFE respectively) was chosen. Polytetrafluoroethylene is considered completely inert and non-degradable (14,15).

The inflammatory response was quantified by characterizing and quantifying the cells involved, both using histo-morphological criteria and monoclonal antibodies directed against epitopes which are fairly specific for the respective cell types. It is difficult to characterize and quantify the cells involved in the inflammatory response when only histo-morphological criteria are used. In particular, this is the case when the cells involved are present in relatively small numbers. The use of monoclonal antibodies permits an accurate characterization and quantification of the cells involved in the inflammatory response.

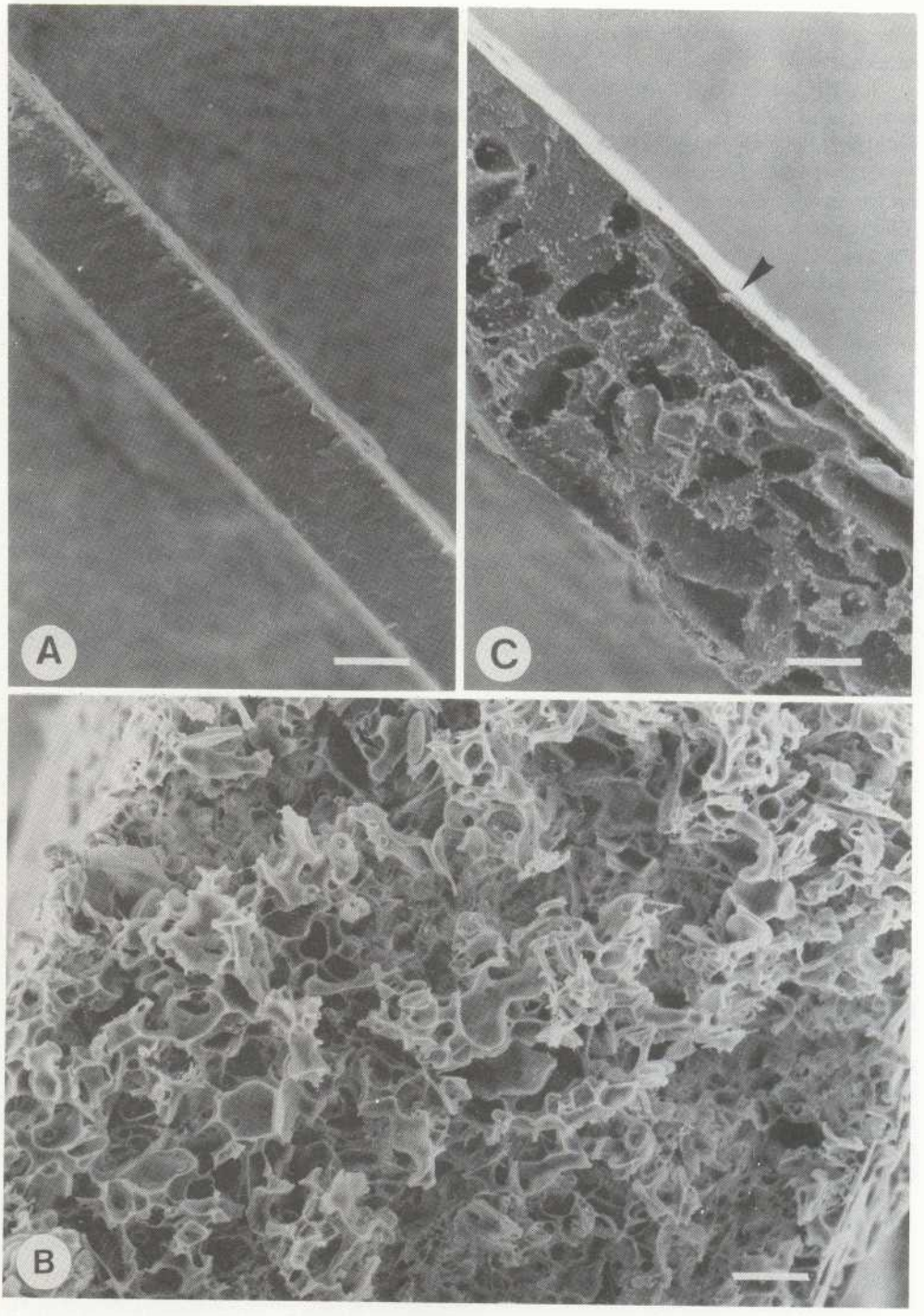
In this study, the influence of morphology and wettability of degradable and non-degradable polymer films on the inflammatory response was investigated by characterizing and quantifying the cells involved in this response.

5.2 Materials and methods

5.2.1 Materials

Poly(L-lactic acid) (PLLA) films were cast from PLLA with a reported Mv of 50,000 (Purac Biochem B.V., The Netherlands)(16). Three types of films were cast: a non-porous type, a porous type and a "combi" type (porous with a non-porous layer on one side).

The **non-porous** type was made using a 20% w/w PLLA solution in chloroform (pro analysi, Merck, Germany) at room temperature. Prior to casting, the solution was not stirred for an hour to allow for the escape of the larger air bubbles. The surface of the glass plate on which the films were cast was cleaned with ethanol (technical grade, distilled 1x). A uniform thickness was obtained by using a Doctor's blade. The distance between the blade and the glass plate was 400 μm. After casting, the chloroform was evaporated in a continuous


A
C
B

flow of dry N_2. The films were removed from the glass plate after 20 hours and washed in ethanol (pro analysi, Merck, Germany) for 16 hours. After drying in air, the films were further dried in vacuum over P_2O_5 at 40 °C.

The **porous** films were made using a 5% w/w PLLA solution in chloroform at room temperature. Subsequently, 40 g sodium citrate (Fluka Chemie, The Netherlands), which was sieved (grain diameter < 36 μm), was added in portions of 10 g to approximately 70 ml PLLA/chloroform solution in order to achieve a film having an approximately 80% pore volume. The salt grains were dispersed in a low capacity ultrasonic bath in order to prevent heating of the solution. Air bubbles were eliminated simultaneously by this procedure. The distance between the blade and the glass plate was 750 μm, controlling the thickness of the solution cast. The films were then dried as described above. After drying, the films were washed in demineralized water for 16 hours in order to remove the salt and subsequently washed for 16 hours in ethanol. The drying procedure which followed was the same as described for the non-porous film.

The **"combi"** films were made by casting a non-porous film as described above on a glass plate first. Subsequently, over this non-porous part of ultimate "combi" film, a porous part was cast as described for the porous film. The distance between the blade and the glass plate was 750 μm. The procedures for washing and drying of these films were as described for the porous films.

All PLLA films were cut in strips of 15x2 mm.

Polytetrafluoroethylene (PTFE) was obtained commercially (Wientjes, The Netherlands). PTFE was cut into strips of 15x2x1 mm.

Expanded polytetrafluoroethylene (ePTFE) was obtained as non-sterile GORE-TEX^R ePTFE cell collector tubing (WL Gore & Associates GMBH, Germany). The tubing was cut open along the longitudinal axis to obtain film measuring 15x2 mm.

Except for PTFE, the final thickness of the films was determined with scanning electron microscopy (SEM). All films were cleaned by washing in a phosphate buffered saline (PBS) for 24 hours prior to implantation.

5.2.2 Morphology

Specimens of all films were sputter-coated with gold (Balzers 07 120B) and their morphology was examined with a DS 130 scanning electron microscope (SEM)(ISI), operated at 10 kV.

Fig. 5.1.

Scanning electron micrographs of a cross-section of PLLA films. A: non-porous, B: porous, C: "combi", arrow head indicates the non-porous side. The thickness of the film is: non-porous, 33 μm, porous, 244 μm and "combi", 82 μm. The non-porous layer of the "combi" film was approximately 5 μm. The pore size of the porous film varied from approximately 1 to 150 μm and of the "combi" film from 1 to 50 μm.
Bar indicates 22 μm.

Fig. 5.2.

Scanning electron micrograph of (porous) ePTFE. The fibril length is approximately 90 μm (= distance between A and B).

5.2.3 Wettability

Wettability was determined by contact angle measurements using the sessile drop technique at 22°C according to the method described by Busscher et al.(17). For PTFE and the non-porous PLLA, the contact angles were determined using H_2O and α-bromonaphthalene as wetting agents.

5.2.4 Cytotoxicity

To detect possible cytotoxicity prior to implantation, films were tested in a direct contact test using a human skin fibroblasts cell line. The fibroblasts were cultured in 25 cm^2 T-Flasks (Greiner, The Netherlands) using RPMI 1640 medium (Gibco Europe B.V., The Netherlands) supplemented with 15% fetal calf serum (Gibco) and 100 IU/ml penicil-

lin/streptomycin (Sigma)(Brunswick, The Netherlands) in humidified air with 5% CO_2 at 37 °C. Every two days, the cultures were subdivided, using trypsin 0.05% in Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS).

The films were disinfected by immersion in ethanol 70% for 1 minute and air-dried under sterile conditions in a laminar flow cabinet. Subsequently, polymer films were mounted on the bottom of a well of a six wells tissue culture polystyrene plate (Greiner). Three ml medium, containing approximately 5.10^4 cells, was added to each well. One well without polymer film (tissue culture polystyrene) served as a negative control. The medium was changed every fourth day.

Cell cultures were evaluated qualitatively for signs of cell lysis, formation of an inhibition zone and/or change in cell morphology for up till 12 days.

5.2.5 Implantation procedure

Prior to implantation, the films were disinfected by immersion in 70% ethanol for 1 minute and air-dried. The films were implanted subcutaneously in 21 female $(AOxBN)F_1$ rats, each weighing approximately 200 g and obtained from our own breeding facility. The rats were anaesthetized with ether, shaved on the back and disinfected with chlorohexidin 0.5% in 70% ethanol. Six incisions of approximately 1 cm were made; three on either side of, and perpendicular to the midline. On each side, incisions were placed parallel to, and at such a distance from each other, in order to prevent the individual inflammatory response against a films to influence the ones against its neighbouring films. Subsequently, subcutaneous pockets were made using blunt scissors. A sample of each type of polymer film was placed in a pocket. Each wound was closed using silk 3-0 (Ethicon) sutures. Thus, in each rat a non-porous PLLA film, a porous PLLA film, a "combi" PLLA film, a PTFE film and an ePTFE film was implanted. The sixth incision and subcutaneous pocket served as a control (SHAM operation). Separate samples were implanted for the various evaluation techniques used in this study. The rats had free access to standard rat food and water. All national rules concerning the care and use of laboratory animals have been observed.

The rats were sacrificed after 1, 3, 7, 14, 40, 90 and 180 days and the polymer films were removed with excess surrounding tissue.

5.2.6 Inflammatory response

Light microscopy: After harvesting, the samples were immediately fixed for at least 24 hours at 4 °C in a 0.1 M Na-cacodylate buffer, pH 7.4, containing 2% glutaraldehyde and 0.1 M sucrose. The samples were then dehydrated in a graded ethanol series and embedded in glycolmethacrylate (Technovit®, Kulzer, Germany), in a position that allowed sections to be cut perpendicular to the longitudinal axis of the polymer film. Subsequently, sections for lightmicroscopical examination were cut on a Jung 1140 autocut microtome, using a D knife with a tungsten carbide cutting edge. Sections were mounted on glass and stained with toluidine blue and alkaline fuchsin (18).

Tab.1.

Monoclonal antibodies used to examine and quantify the inflammatory response against the implanted polymer films.

Monoclonal Antibody	Epitope	Mainly characterizing cell type in subcutaneous tissue:	Ref.no.
OX 1	CD 45	All leucocytes	18
HIS 48	surface antigen?	Granulocytes	19
OX 19	CD 5	T-lymphocytes	20
HIS 40	IgM heavy chain	B-lymphocyte subset likely to react first upon inflammatory response	21
ED 1	Lysosomal antigen	Majority of macrophages. Association with active phagocytosis?	22
ED 2	Surface antigen	Subset macrophages. Association with maturity?	22, 23
ED 3	Surface antigen	Subset macrophages. Associated with downregulation of inflammatory reaction?	22, 24
α-Asialo GM1	surface antigen?	Large granular lymphocytes/natural killer cells	25
HIS 19	MHC class II	Activated tissue cells (fibroblast) dendritic cells, IDC, Subset macrophage	26

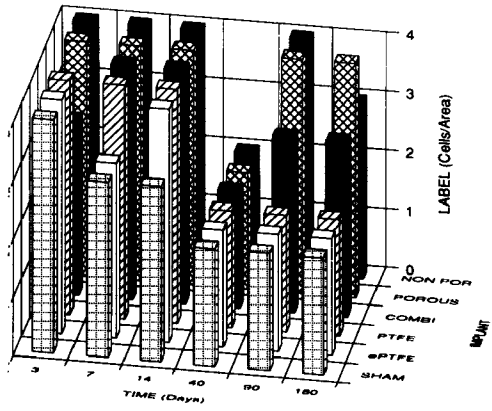
Immunohistochemical staining: After harvesting, samples were snap-frozen at -80 °C using liquid freon. Cryostat sections of 7 μm were cut, mounted on glass slides, air-dried and fixed in acetone for 12 minutes. The sections were then again air-dried for 1 hour and incubated with the first-stage, cell type specific, mAb for 1 hour. Subsequently, the sections were washed 3 times in PBS, followed by incubation with the second-stage antibody conjugated to peroxidase, diluted 1:40 in PBS and supplemented with 5% v/v normal rat serum to prevent nonspecific binding. Swine anti-rabbit Ig (Dakopatts, Denmark) was used as second-stage antibody to detect the first-stage mAb α-Asialo GM_1 (Tab.1). Rabbit anti-mouse Ig (Dakopatts, Denmark) was used to detect the other first-stage mAbs. After incubation with the second-stage antibody, sections were rinsed 3 times in PBS for 5 minutes. Peroxidase activity was demonstrated by applying 3,3'-diaminobenzidine tetrahydrochloride (Sigma) at a concentration of 0.5 mg/ml in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% H_2O_2 for 10 minutes. After rinsing in fresh tap water, sections were counterstained lightly with haematoxylin for 10 seconds. The sections were then dehydrated using a graded ethanol series and xylene and subsequently covered with coverslips using DePeX (Gurr, BDH Ltd, England) mounting medium. In controls, PBS was used instead of the first-stage mAb.

Quantification of the inflammatory response: The magnification of the light microscope was set at 400x. The staining patterns of the tissue surrounding or invading the polymer film was then examined and the number of positive cells surrounding or invading the polymer films per field of view counted. At least four fields per section and at least two sections were examined for each period of implantation, polymer film and monoclonal antibody respectively. Sections at the edges of the polymer films were excluded from evaluation, to avoid artifacts due to mechanical irritation.
The number of cells staining positively was classified as follows:
grade **0** = no positive cells,
1 = 1 to 5 positive cells per field of view,
2 = 5 to 10 positive cells per field of view,
3 = 10 to 25 positive cells per field of view and
4 = more than 25 positive cells per field of view.
For the monoclonals used, their respective specificity and relevant references see Tab.1.

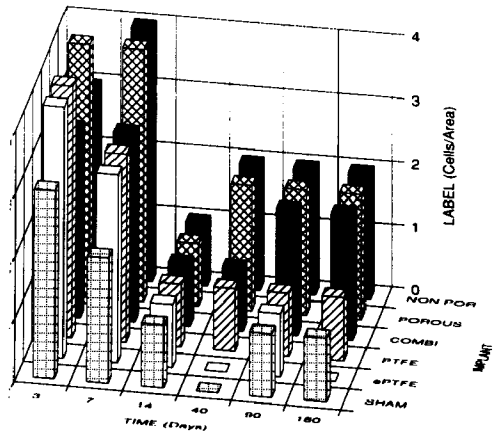
5.3 Results

5.3.1 Morphology

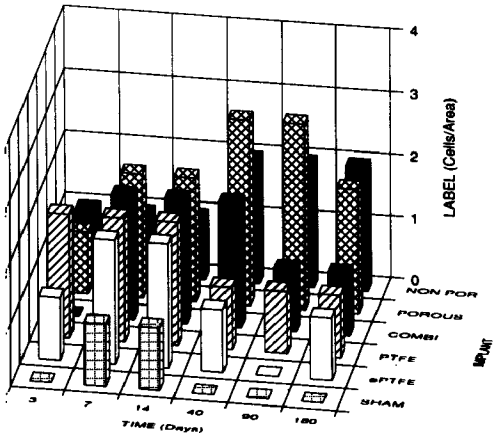
The morphology of the respective PLLA films was demonstrated by scanning electron microscopy. Their thickness was calculated: non-porous, 33 μm (Fig. 5.1A), porous, 244 μm (Fig. 5.1B) and the "combi" film, 82 μm (Fig. 5.1C). The non-porous layer of the "combi" film was approximately 5 μm. The pore size of the porous film varied from approximately 1 to 150 μm and of the "combi" film from 1 to 50 μm . PTFE was non-porous, having the same morphological appearance as the non-porous PLLA film. The thickness of the porous



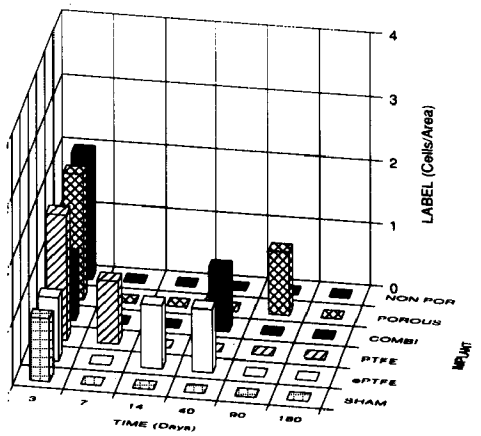
A ALL LEUCOCYTES (OX 1)



B NEUTROPHILS (HIS 48)



C T-LYMPHOCYTES (OX 19)



D B-LYMPHOCYTES (HIS 40)

Fig. 5.3.

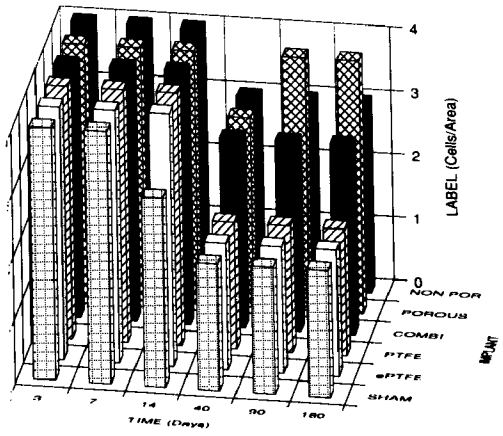
Classification of the number of cells involved in the inflammatory response against the implanted polymer films using different mAbs. Classification criteria: grade 0 = no positive cells, grade 1 = 1 to 5 positive cells, grade 2 = 5 to 10 positive cells, grade 3 = 10 to 25 positive cells and grade 4 = more than 25 positive cells per field of view at a original magnification of 400x.

The following mAbs were used: A: OX 1, all leucocytes, B: HIS 48, neutrophilic granulocytes, C: OX 19, T-lymphocytes, D: HIS 40, vast majority of B lymphocytes, E: ED 1, vast majority of macrophages, including multinuclear giant cells, F: ED 2, subset (mature/resident) macrophages, G: ED 3, subset macrophages, H: α-Asialo GM_1, natural killer cells/large granular lymphocytes, I: HIS 19, cells expressing MHC II antigen, e.g. activated fibroblasts, macrophages, dendritic cells)

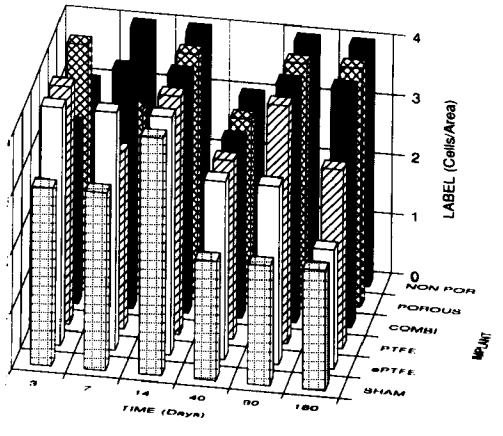
X-axis represents implantation time (days)

Y-axis represents number of cells per field of view.

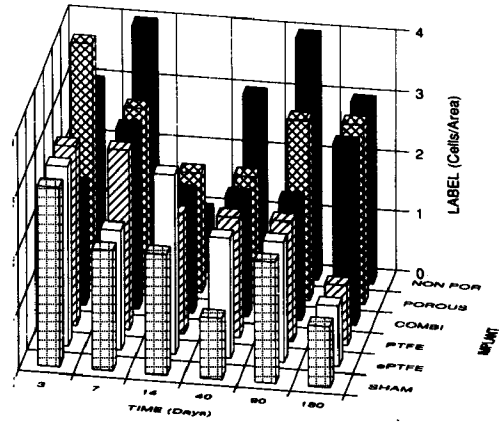
Z-axis represents the different polymer films.



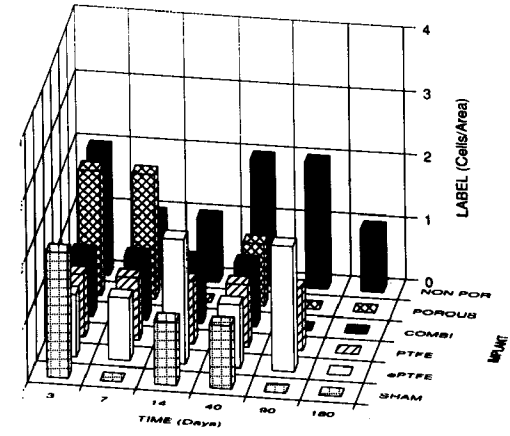
E MACROPHAGES (ED 1)



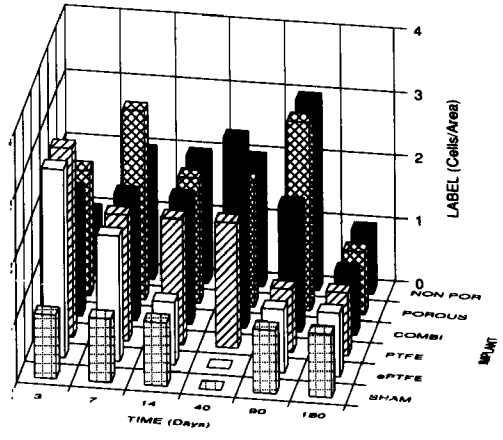
F MACROPHAGES (ED 2)



G MACROPHAGES (ED 3)



H N K CELLS (ASIALO)



I MHC II ANTIGEN (HIS 19)

ePTFE film was approximately 250 μm, with a fibril length of approximately 90 μm (Fig. 5.2).

5.3.2 Wettability

The H_2O contact angles were 101° for PTFE and 72° for PLLA and the α-bromonaphthalene contact angles were 66° for PTFE and 23° for non-porous PLLA. This shows that non-porous PLLA has a less hydrophobic surface than PTFE.

5.3.3 Cytotoxicity

Neither cell death, nor the formation of cell growth inhibition zones were observed. The morphological appearance of the fibroblasts in all wells in which materials were tested was normal and not differing from the controls. Thus there was probably no release of large quantities of toxic products up till the end of the experiment at day 12.

5.3.4 Inflammatory response

The results of the semi-quantitative evaluation of the immunohistochemical staining to detect leucocytes (OX 1), neutrophilic granulocytes (HIS 48), macrophages (ED 1, ED 2, ED 3), T-lymphocytes (OX 19), vast majority of B-lymphocyte (HIS 40), natural killer cells (α-asialo GM_1), cells expressing MHC class II antigen, such as activated fibroblasts (His 19) are demonstrated in Fig. 5.3A to Fig. 5.3I respectively. In PBS controls, occasionally, false positive cells were observed, due to endogenous peroxidase activity of the cell. The endogenous peroxidase activity is expressed by hardly any other cell type but neutrophilic granulocytes, since the positive staining pattern with HIS 48 corresponds with the pattern of endogenous peroxidase activity. Moreover, cells expressing endogenous peroxidase activity can be well characterized, due to their intense staining as compared to cells stained for mAbs.

The number of B-lymphocytes (Fig. 5.3D) and the number of natural killer cells (Fig. 5.3H) was to a large extent similar and low for both PLLA, PTFE films and the subcutaneous tissue under the scar of the SHAM operation.

At **day 3**, light microscopical examination demonstrates that the majority of the cells surrounding the polymer films were granulocytes and monocytes/macrophages. The amount of each cell type involved in the inflammatory response were approximately the same for all the polymers films implanted (Fig. 5.3A to 5.3G). Apparently, the intensity of the inflammatory response against the polymer films is not very different.

At **day 7**, it is demonstrated that macrophages are beginning to play a prominent role in the inflammatory response, both in GMA sections and using immunohistochemical staining with ED1 (Fig. 5.3E). Subsets of macrophages surrounding the strip of polymer films were found in different locations. ED 1 positive macrophages are found in the entire inflammatory area surrounding the implant, especially in the area in closest approximation of the polymer

film (Fig. 5.4). In contrast, ED 2 and ED 3 positive macrophages are neither found in the inflammatory area next to the polymer film, nor in the pores of the PLLA or PTFE film (Fig. 5.5 and 5.6). ED 2 and ED 3 positive macrophages remain restricted to the surrounding tissue, at some distance from the implant. The relative ratio between the subsets was fairly constant for the respective implants.

The difference in the intensity of the inflammatory response between the different polymer films is more pronounced as compared to day 3. The difference, however, remains small. However, in GMA sections it is observed that the inflammatory response at day 7 is becoming more intense (Fig. 5.7) for the porous and porous side of the "combi" PLLA films. For the non-porous PLLA and the non-porous side of the "combi" PLLA film, the onset of encapsulation by approximately 3 layers of fibroblasts was observed. In contrast, neutrophils invaded the porous PLLA film. Only a minimal encapsulation is observed for the porous PLLA film at day 7.

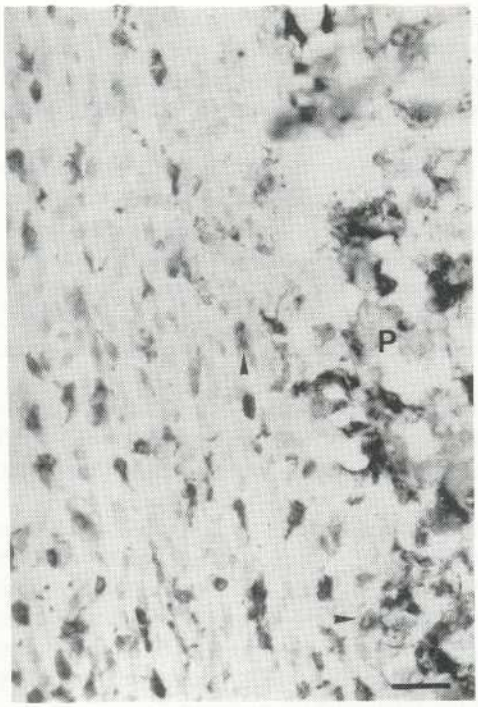
There was almost no cellular invasion of the ePTFE film when compared to the porous PLLA film. The cell layer surrounding ePTFE consists mainly of macrophages. The PTFE film was surrounded by one or two layers of macrophages and the onset of encapsulation can be observed.

At **day 14**, the intensity of the inflammatory response decreased as illustrated in Fig. 5.3A to 3I. The number of leucocytes and fibroblasts decreases to the level observed in the subcutaneous tissue under the scar of the SHAM operation. The number of macrophages and T-lymphocytes remains high. In GMA sections, foreign body giant cells can be observed surrounding the PLLA films (Fig. 5.8). All films are now encapsulated by continuous layers of fibrocytes and collagen. The fibrocytes are not stained by HIS 19 monoclonal antibody, indicating a decrease in cell activity related to the inflammatory response.

Again, using GMA sections, a small difference in the distribution pattern of the macrophages was observed between the non-porous PLLA film and non-porous side of the "combi" film on the one hand and the porous PLLA film and porous side of the "combi" film on the other: porosity induces an increase in the number of macrophages surrounding the PLLA films. However, this observation could not be made for the ePTFE film as compared to the PTFE film.

At **day 40**, in general, the number of cells involved in the inflammatory response against the polymer films had decreased further, especially for the PTFE and ePTFE films. The degradable PLLA films provoked a more intense inflammatory response as compared to the PTFE films. Generally, the number of neutrophils, macrophages/giant cells and T-lymphocytes surrounding the strips of PLLA films had increased (Fig. 5.3B, 5.3C, 5.3E, 5.3F and 5.3G).

The inflammatory response against the non-porous PLLA film, mainly consisted of giant cells and macrophages and was mainly localized at the edges of the film or at places where the film was fragmented. In contrast, the inflammatory response against porous PLLA films, also mainly consisting of giant cells and macrophages, is localized in the pores. The "combi" film shows a more pronounced inflammatory response at the porous side, but in general, the intensity of the tissue reaction is less when compared to the non-porous and porous PLLA film.



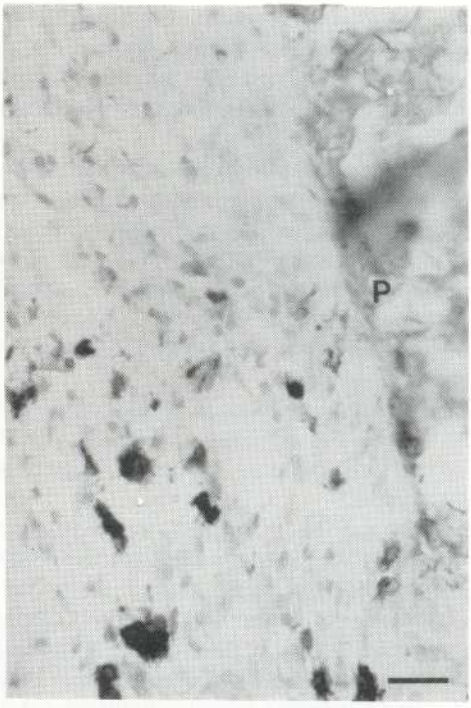


Fig. 5.4. (top left)

Immunohistochemical staining of the tissue surrounding the porous PLLA film (P) with ED 1, 7 days after implantation. Note that the macrophages adjacent to the implant stain positive for ED 1 (arrow heads). No ED 1 positive cells are observed in the polymer film. Bar indicates 40 μm.

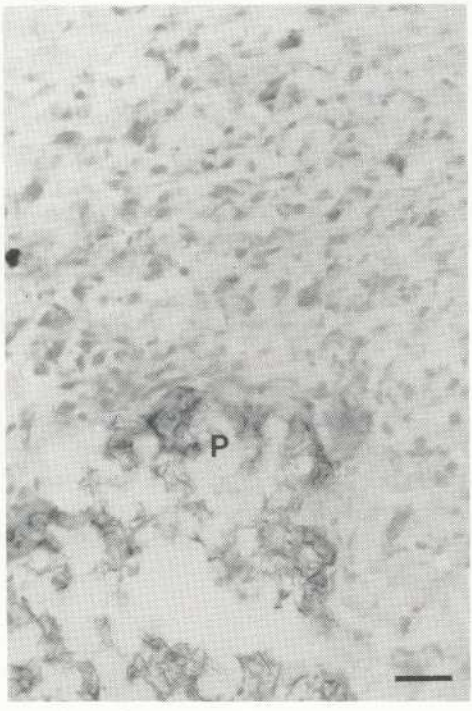


Fig. 5.5. (top right)

Immunohistochemical staining of the tissue surrounding the porous PLLA film (P) with ED 2, 7 days after implantation. Note that the macrophages adjacent to the implant do not stain positive for ED 2. The cells in the left lower corner stain false positive for ED 2. This is due to endogenous peroxidase activity of neutrophilic granulocytes. Bar denotes 40 μm.

Fig. 5.6. (bottom left)

Immunohistochemical staining of the tissue surrounding the porous PLLA film (P) with ED 3, 7 days after implantation. Note that the macrophages adjacent to the implant do not stain positive for ED 3. Bar indicates 40 μm.

The number of cells involved in the inflammatory response against PTFE and ePTFE is comparable to the number of cells in the subcutaneous tissue under the scar of the SHAM operation.

At **day 90**, the difference in the intensity of the inflammatory response between PLLA and PTFE films and also between non-porous and porous PLLA films had become much more pronounced. All films were encapsulated by fibrous connective tissue. The number of macrophages and leucocytes surrounding or invading the PLLA films had increased and the number of cells expressing MHC class II (HIS 19) antigen, (probably activated fibroblasts) increased. The porous PLLA films provokes a more intense inflammatory response, as compared to the non-porous PLLA film. This inflammatory response was mainly localized in the pores.

In contrast, the inflammatory response against PTFE and ePTFE films remained stable after day 40.

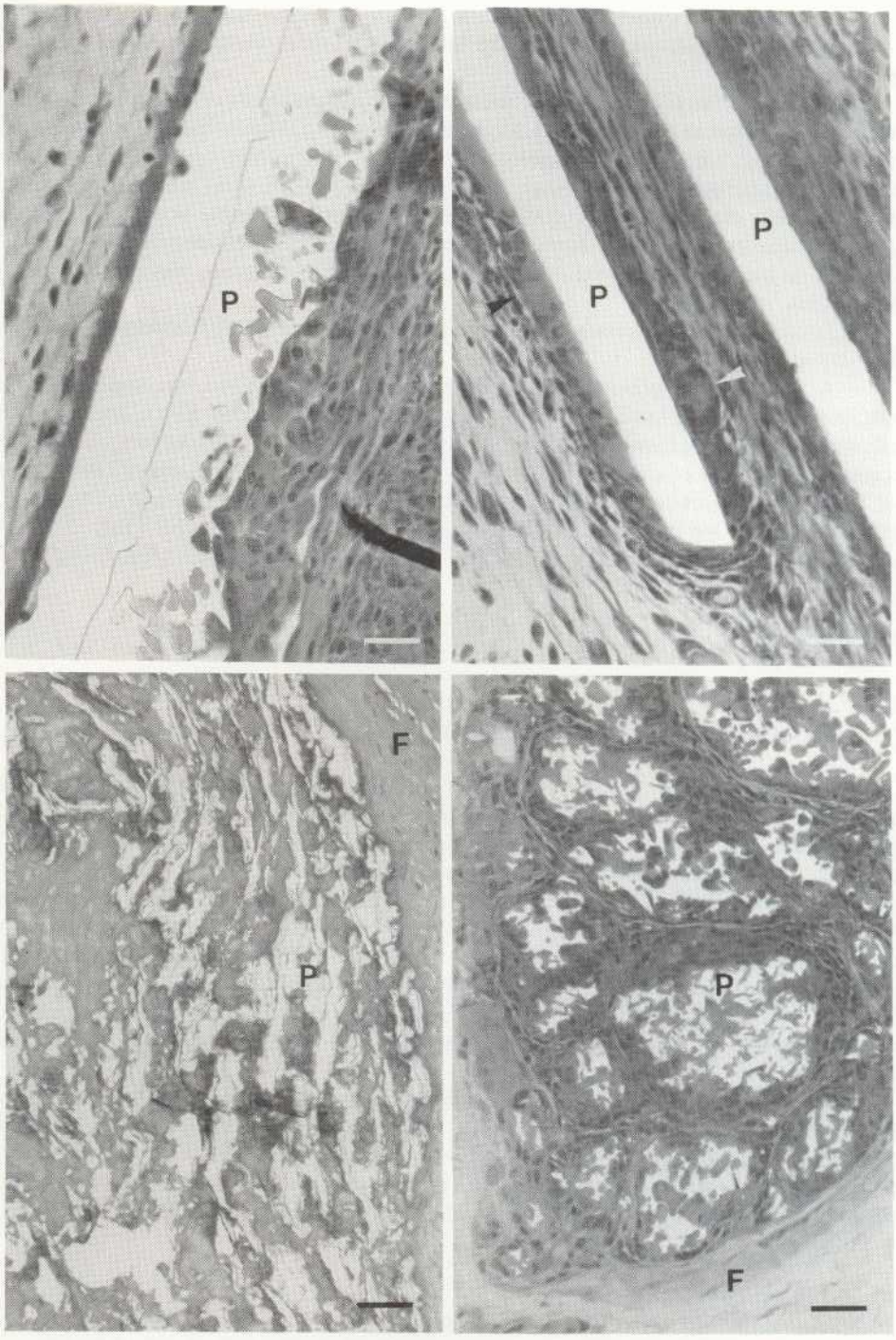
At **day 180**, the inflammatory response against the PLLA films was consisted of giant cells, macrophages and small numbers of neutrophils. The difference in the intensity of the inflammatory response between the non-porous and porous PLLA film was more pronounced. In contrast, the tissue reaction ("inflammatory response") against PTFE and ePTFE did again not differ from the tissue reaction in the subcutaneous tissue under the scar of the SHAM operation. There are almost no cells localized in the pores of ePTFE (Fig. 5.9). The inflammatory response against porous PLLA was localized in the pores (Fig. 5.10).

5.4 Discussion

5.4.1 Inflammatory response and biocompatibility

The results demonstrate that two phases can be recognized in the inflammatory response against the films. Phase 1 is observed upon implantation of a biomaterial. It was mainly caused by the trauma sustained by the implantation procedure. This uncomplicated inflammatory response, part of the wound healing reaction, ends after 7 to 10 days and has been well described (2,28). In this phase, the contribution of the implanted polymer film to intensity of the inflammatory response is in most cases minimal, except when leakage of large quantities of toxic products occurs (29). In this study, at day 14, no differences in the intensity and nature of the inflammatory response between PLLA (degradable) and PTFE (non degradable) films were observed. It appears that the duration acute inflammatory response against the polymer films is only slightly extended compared to the one which is part of the wound healing reaction.

After one week, any remaining inflammatory response can be considered as a tissue reaction against the implanted biomaterial (30). This chronic (phase 2) inflammatory response is often described as a foreign body reaction (1,2,30). It mainly consists of macrophages and giant cells surrounding the implant. The implant is then encapsulated by a relatively thin fibrous tissue layer. Many authors do not consider the presence of a foreign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body reaction to be an unfavourable sign in terms of biocompatibility. Nevertheless, many authors prefer the situation where the implanted material is only surrounded by an "avascular and almost acellular fibrous encapsulation" (1,2). The relation between chronic inflammatory response and amyloidosis (31) or carcinogenesis (32) is strongly suggested. Therefore, one must aim at a minimal inflammatory response, especially when biomaterials are implanted for a long time span.

In general, biodegradable materials provoke the same inflammatory response as described above. However, upon degradation, the release of degradation products such as monomers, oligomers and fragments may cause a renewed or aggravated inflammatory response (10,30). Moreover, the mere change of shape is probably enough to increase the intensity of the tissue reaction. This renewed inflammatory response often has a more "acute" aspect, represented by a higher number of neutrophilic granulocytes, involved in the inflammatory response.

5.4.2 The role of macrophages and giant cells

ED 2 and ED 3 do not stain macrophages or giant cells directly adjacent to the implanted polymer film, indicating that: 1. There are different populations of macrophages which play a role in the tissue reaction against biomaterials, or 2. macrophages express different surface receptors in the course of the inflammatory response against a biomaterial. ED 2 is a marker which is found on resident tissue macrophages. It takes about one week for monocytes/macrophages to express ED 2 on their cell surface after leaving the vascular system (24). It is most likely that ED 2 positive macrophages are partly resident macrophages and partly matured from blood monocytes. ED 1 stains an intracellular antigen probably associated with the lysosomal membrane (23). The antigen probably remains present, even in the event

Fig. 5.7. (top left)

"Combi" PLLA film (P) 7 days after implantation. Note the difference in the intensity of the inflammatory response between the porous and non-porous side. Bar indicates 40 μm.

Fig. 5.8. (top right)

Non-porous PLLA film (P) 14 days after subcutaneous implantation. Note the large amounts of giant cells (arrow heads). Bar denotes 40 μm.

Fig. 5.9. (bottom left)

Porous ePTFE (P) after 180 days of implantation. There are almost no inflammatory cells localized in the pores. F = fibrous encapsulation. Bar indicates 63 μm.

Fig. 5.10. (bottom right)

Porous PLLA (P) after 180 days of implantation. The inflammatory response is mainly localized in the pores. F = fibrous capsule. Bar indicates 63 μm.

of fusion of macrophages and formation of foreign body giant cells, since ED 1 positive cells are observed on the same location of foreign body giant cells in the conventional light microscopical sections. Apparently, the ED 2 antigen is lost during prolonged activation of macrophages, as ED 2 positive cells are never observed near the implanted biomaterial. The same goes for ED 3 positive macrophages, although their role in the inflammatory response is even less understood (25).

The exact mechanism of foreign body giant cell formation is still to be elucidated. Certain cell types, *e.g.* T-lymphocytes, may play a pivotal role in this process. Especially T-helper lymphocytes capable of secreting interferon-γ may play a role in the fusion of macrophages, forming giant cells (33,34). Activation of macrophages may be induced by various pathways. One pathway may be (limited) damage to neutrophils and macrophages upon which cytokines (IL-1) may be secreted to activate T-helper lymphocytes. Another possibility is change of shape when a macrophage comes in contact with a biomaterial (35,36), especially when a single macrophage is not able to span the foreign body.

5.4.3 Porosity, wettability, degradability and inflammatory response

The differences in the inflammatory response against non-porous and porous PLLA becomes apparent from day 7 on (Fig. 5.7), indicating that porosity is an important factor determining the intensity of the inflammatory response against implanted PLLA films. Till day 7, the inflammatory response appears to be the same against all the polymer films.

The small difference in the intensity of the tissue reaction between PTFE and ePTFE as compared to non-porous and porous PLLA indicates that difference in porosity is not enough to account for all the differences in the inflammatory response. As demonstrated, PLLA is more wettable than PTFE. This higher wettability can cause a the better ingrowth of tissue into the pores of PLLA and subsequently more exposure to other factors, triggering an inflammatory response.

The differences in the inflammatory response against the PLLA (degradable) and PTFE (non degradable) films becomes apparent from day 40 on. The main reason is most likely the difference in the rate of degradation and subsequently the difference in the release of degradation products, such as monomers, oligomers and, in the later stages, fragments. However, difference in wettability may also be a factor, although Baier et al. using smooth metal pieces having a different wettability could not demonstrate a difference in the inflammatory response against them (37).

5.5 Conclusions

In summary, it can be concluded that degradable PLLA films provoke a more intense inflammatory response than non degradable PTFE films due to their degradability and perhaps higher wettability. Porous PLLA provokes a more intense inflammatory response than non-porous PLLA. However, it is also appears that the wettability of a biomaterial has to be high enough to allow for porosity to be a cause of a more intense inflammatory response.

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